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AUTHOR(S):

Suzuki, Shinnosuke; Nozawa, Yusuke; Tsukamoto, Satoshi; Kaneko, Takehito; Imai, Hiroshi; Minami, Naojiro

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Histone methyltransferase *Smyd3* regulates early embryonic lineage commitment in the mouse

Shinnosuke Suzuki[1,*], Yusuke Nozawa[1,*], Satoshi Tsukamoto[2], Takehito Kaneko[3], Hiroshi Imai[1], Naojiro Minami[1]

[1] Laboratory of Reproductive Biology, Graduate School of Agriculture, Kyoto University, Kyoto, Japan.

[2] Laboratory of Animal and Genome Sciences Section, National Institute of Radiological Sciences, Chiba 263-8555, Japan.

[3] Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University, Kyoto, Japan.

Correspondence should be addressed to N Minami; E-mail: oog1nao@kais.kyoto-u.ac.jp

[*] These authors contributed equally to this work.

Short title: Role of *Smyd3* in embryonic lineage commitment

Abstract

Smyd3 (SET and MYND domain–containing protein 3) is a histone H3 lysine 4 (H3K4) di- and tri-methyltransferase that forms a transcriptional complex with RNA polymerase II and activates the transcription of oncogenes and cell cycle genes in human cancer cells. However, the study of *Smyd3* in mammalian early embryonic development has not yet been addressed. In the present study, we investigated the expression pattern of *Smyd3* in mouse preimplantation embryos and the effects of RNA interference (RNAi)-mediated *Smyd3* repression on the development of mouse embryos. Here, we showed that *Smyd3* mRNA levels increased after the 2-cell stage, peaked at the 4-cell stage, and gradually decreased thereafter. Moreover, in 2-cell to 8-cell embryos, SMYD3 staining was more intense in the nuclei than in the cytoplasm. In *Smyd3*-knockdown embryos, the percentage of inner cell mass (ICM)-derived colony formation and trophectoderm (TE)-derived cell attachment was significantly decreased, resulting in a reduction in the number of viable offspring. Furthermore, the expression of *Oct4* and *Cdx2* during mid-preimplantation gene activation was significantly decreased in *Smyd3*-knockdown embryos. In addition, the transcription levels of ICM and epiblast markers, such as *Oct4*, *Nanog*, and *Sox2*; of primitive endoderm markers, such as *Gata6*; and of TE markers, such as *Cdx2* and *Eomes*, were significantly decreased in

Smyd3-knockdown blastocysts. These findings indicated that SMYD3 plays an important role in early embryonic lineage commitment and peri-implantation development through the activation of lineage-specific genes.

Introduction

Embryonic development in mammals is characterized by an initial preimplantation phase that serves to prepare the embryo for implantation. Transcription from the newly formed zygotic genome, known as zygotic gene activation (ZGA), begins after fertilization between the late 1-cell stage and the 2-cell stage (Latham and Schultz 2001, Li, et al. 2010, Schultz and Worrad 1995). Subsequently, mid-preimplantation gene activation (MGA) occurs during the 4- to 8-cell stages (Hamatani, et al. 2004). Both ZGA and MGA consist of new gene expression from the embryonic genome, and both steps require proper lineage commitment and differentiation. The first lineage differentiation gives rise to the inner cell mass (ICM) and trophectoderm (TE). The pluripotency of the ICM lineage is regulated by the transcription factors *Oct4* (also known as *Pou5f1*), *Nanog*, and *Sox2* (Avilion, et al. 2003, Mitsui, et al. 2003, Nichols, et al. 1998), and the specification and differentiation of the TE lineage is regulated by the transcription factors *Cdx2* and *Eomes* (Russ, et al. 2000, Strumpf, et al. 2005). Prior to implantation, the ICM gives rise to the epiblast (EPI), which predominantly expresses *Nanog*, and the primitive endoderm (PE), which predominantly expresses *Gata6* (Chazaud, et al. 2006, Rossant 2004). The EPI will eventually give rise to the fetus, while the PE will develop into the visceral and parietal endoderm of the yolk sacs, and the TE will become

the fetal placenta.

In general, gene expression is regulated through the transition of several epigenetic factors, including transcription factors, chromatin-remodeling factors, and some enzymes. Examples of epigenetic changes that take place during preimplantation development include DNA methylation, histone post-translational modifications, and histone variant exchange (Akiyama, et al. 2011, Hirasawa, et al. 2008, Santos, et al. 2005, Sarmento, et al. 2004). Drastic changes in many varieties of histone post-translational modifications occur during ZGA. Histone post-translational modifications are introduced in a variety of ways. Several enzymes contribute to histone methylation (Zhang and Reinberg 2001), acetylation (Sterner and Berger 2000), phosphorylation (Nowak and Corces 2004), and ubiquitination (Shilatifard 2006). With respect to methylation, modifications of lysines 4, 36, and 79 of histone H3 (referred to as H3K4, H3K36, and H3K79, respectively) are associated with transcriptional activation, whereas modifications of lysines 9 and 27 of histone H3 and lysine 20 of histone H4 (referred to as H3K9, H3K27, and H4K20, respectively) are associated with transcriptional repression (Lepikhov and Walter 2004, Sarmento, et al. 2004). Except for the H3K79 methyltransferase, known as DOT1L, histone methyltransferases (HMTase) include a conserved catalytic domain called the SET domain (Feng, et al. 2002, Zhang and Reinberg 2001). *Smyd3* (SET and MYND domain containing protein 3)

encodes a protein comprising 428 amino acids and containing a SET-domain, a MYND-type zinc finger domain, and a SET-N region. SMYD3 has been reported to be capable of methylating both H3K4 and H4K5 (Hamamoto, et al. 2004, Van Aller, et al. 2012). Evidence has accumulated that SMYD3 recruits RNA polymerase II through an RNA helicase to form a transcription complex, and that it elicits its oncogenic effects by activating the transcription of downstream target genes (Hamamoto, et al. 2004, Hamamoto, et al. 2006, Liu, et al. 2007, Liu, et al. 2013). Previous reports have demonstrated that enhanced expression of SMYD3 is essential for the growth of human cancer cells (Hamamoto, et al. 2004, Hamamoto, et al. 2006), whereas the suppression of SMYD3 expression leads to apoptosis and the inhibition of cell growth, migration, and invasion (Chen, et al. 2007, Zou, et al. 2009). Recent studies have determined that *Smyd3* plays an important role in the development of heart and skeletal muscle during zebrafish embryogenesis (Fujii, et al. 2011). However, the role of SMYD3 in mammalian early embryonic development has not been previously addressed.

Here, we examined the expression pattern of *Smyd3* mRNA and the localization of SMYD3 protein in mouse preimplantation embryos, and found that siRNA-mediated knockdown of *Smyd3* during early stages of embryonic development suppressed *Oct4* and *Cdx2* at MGA. Additionally, *Smyd3* knockdown early in development reduced the blastocyst-stage expression of

ICM/EPI markers, e.g., *Oct4*, *Nanog*, and *Sox2*; of PE markers, e.g., *Gata6*; and of TE markers, e.g., *Cdx2* and *Eomes*. However, the number of apoptotic cells was not increased until Day4 after outgrowth experiments (E7.5) in *Smyd3*-knockdown embryos. From these results, we propose that SMYD3 plays an important role in early embryonic lineage commitment through the activation of lineage-specific genes.

Materials and Methods

In vitro fertilization (IVF) and embryo culture

IVF was performed as previously described (Suzuki, et al. 2015). Three hours after insemination, fertilized eggs were washed and cultured until embryonic day 5.5 (E5.5) in K-modified simplex optimized medium (KSOM) containing 4 mg/mL BSA under mineral oil (Sigma-Aldrich) at 37 °C, in an atmosphere of 5% CO_2 in air or used for microinjection (Tsukamoto, et al. 2013) and cultured until E5.5.

RNA extraction and quantitative RT-PCR (qRT-PCR)

Metaphase II oocytes, and 1-cell, 2-cell, 4-cell, 8-cell, morula, and blastocyst stage

69 embryos were collected at 14, 28, 50, 62, 69, 90 and 122 h after hCG injection, respectively. RNA
70 extraction and qRT-PCR were performed as described (Suzuki, et al. 2013). Total RNA from 30
71 embryos at each stage was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA).
72 Transcription levels were determined using three different sets of 30 embryos per stage and
73 normalized to *H2afz* known as a stable reference gene for normalization of gene expression in
74 mouse preimplantation embryos (Jeong, et al. 2005, Mamo, et al. 2007) or *Gapdh*; relative gene
75 expression was analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). All primers used for
76 PCR are listed in Supplementary Table 1.
77
78 **Microinjection of siRNA**
79 Fertilized embryos transferred to KSOM were microinjected into the cytoplasm with 5–10
80 pL of 100 μM *Smyd3* siRNA (si*Smyd3*-1: 5'-GCAGGGUUAUCGUCAAGCUGA-3'; si*Smyd3*-2:
81 5'-GUCGUGGCGUAGUCUGUGAUC-3'; RNAi Inc., Japan) between 3 and 4 h after insemination.
82 The same amount of negative control siRNA (siControl; RNAi Inc.), which contains scrambled
83 sequences from the si*Smyd3*-1 or si*Smyd3*-2 construct, was also microinjected as a control. To
84 examine the developmental competency and hatching ability, embryos were observed at 50 (E1.5),
85 74 (E2.5), 98 (E3.5), 122 (E4.5) and 146 h (E5.5) after hCG injection. After siRNA injection,

embryos were harvested at either the 4- and 8-cell stages (62 and 69 h after hCG injection, respectively) for qRT-PCR and immunostaining, at the morula or blastocyst stage (98 h after hCG injection) for outgrowth experiments, or at the blastocyst stage (122 h after hCG injection) for qRT-PCR, immunostaining, and immunoblotting.

Outgrowth experiment and embryo transfer

Outgrowth experiments were performed using morula or blastocyst stage embryos collected 98 h after hCG injection as previously described (Yamada, et al. 2010). After culture for 4 days, the percentage of blastocysts that underwent outgrowth was calculated and photographed. A portion of the embryos that reached the 2-cell stage after microinjection was transferred into the oviducts of 0.5 dpc pseudopregnant ICR female mice. These females were sacrificed at Day 19, and pups were counted. The experiment was repeated four times.

Immunostaining

Embryos for immunostaining were collected as described above. For SMYD3, trimethylated H3K4 (H3K4me3), and EOMES staining, embryos were fixed in 4% paraformaldehyde in PBS for 20 min at 4 °C after the removal of the zona pellucidae with Acid

Tyrode's Solution (pH 2.5). After washing three times in PBS containing 0.3% polyvinylpyrrolidone (PVP K-30, Nacalai Tesque, Kyoto, Japan) (PBS/PVP), fixed embryos were treated with 0.5% Triton X-100 (Sigma-Aldrich) in PBS for 40 min at room temperature (RT), and blocked in PBS containing 1% BSA for 1h at RT (for SMYD3 and EOMES) or 3% BSA overnight at 4 °C (for H3K4me3). Next, embryos were incubated overnight at 4 °C with a rabbit anti-SMYD3 antibody (1:100 dilution, 10 µg/mL; ab16027, Abcam Ltd, Cambridge, UK) or rabbit anti-EOMES antibody (1:500 dilution, 0.4 µg/mL; ab23345, Abcam Ltd), or for 1 h at RT with a rabbit anti-H3K4me3 antibody (1:200 dilution, 2.5 µg/mL; ab8580, Abcam Ltd) in antibody dilution buffer (PBS containing 1% BSA). Embryos were washed three times in antibody dilution buffer, and then incubated with the appropriate secondary antibody diluted at 1:500 (Alexa Fluor 488-conjugated goat anti-rabbit IgG or Alexa Fluor 594-conjugated goat anti rabbit IgG, Invitrogen) for 1 h at RT. Immunostaining with normal rabbit IgG (sc-2027, Santa Cruz Biotechnology, Dallas, TX) was performed as a negative control for the specificity of the anti-SMYD3 antibody. For NANOG, SOX2, and GATA6 staining, embryos were fixed, permeabilized, and blocked as previously described (Ralston and Rossant 2008). Next, embryos were incubated overnight at 4 °C with a rabbit anti-NANOG antibody (1:1000 dilution, 1 µg/mL; ab5731, Millipore, Bedford, MA), a goat anti-SOX2 antibody (1:100 dilution, 2 µg/mL; sc-17320, Santa Cruz Biotechnology), or a goat

anti-GATA6 antibody (1:1000 dilution, 0.2 µg/mL; AF1700, R & D systems, Inc., Minneapolis, MN) in blocking solution for overnight at 4 °C. Embryos were washed three times in blocking solution, and then incubated with the appropriate secondary antibody diluted at 1:750 (Alexa Fluor 594-conjugated goat anti-rabbit IgG, Invitrogen) or 1:500 or 1:750 (Alexa Fluor 594-conjugated rabbit anti-goat IgG, Invitrogen) for 1 h at RT. After staining, the samples were washed three times in antibody dilution buffer or blocking solution for 15 min, and nuclei were stained in PBS containing 10 µg/mL Hoechst 33342 (Sigma-Aldrich) for 10 min at RT. Immunofluorescent staining for OCT4 and CDX2 was performed as previously described (Isaji, et al. 2013). After staining, embryos were mounted on slides in 50% glycerol/PBS and SMYD3-related signals were observed using a fluorescence microscope (BZ-X700, Keyence, Osaka, Japan) equipped with structured illumination microscopy (Gustafsson 2005, Hosny, et al. 2013). Fluorescein signals related to OCT4, CDX2, H3K4me3, NANOG, SOX2, GATA6, and EOMES were detected using fluorescence microscopy (BX50, Olympus, Tokyo, Japan). At least 20 samples were examined in each group. The numbers of ICM and TE cells were determined by counting OCT4- and CDX2-positive cells, respectively. To count the cell number and observe the localization of OCT4, CDX2, NANOG, SOX2, GATA6, and EOMES in *Smyd3*-knockdown embryos exposure time was extended compared with that of control embryos. The total embryonic cell numbers were obtained

by adding the numbers determined for the ICM and TE cells.

Immunoblotting

Immunoblotting was performed as previously described (Suzuki, et al. 2013). Total proteins from 100 embryos were extracted using SDS sample buffer/Lysis buffer (1:1). Primary antibody was used; a mouse anti-α-TUBULIN antibody (1:5000 dilution, 1.16 µg/mL; T9026; Sigma-Aldrich), a rabbit anti-SMYD3 antibody (1:100 dilution, 10 µg/mL), a rabbit anti-H3K4me3 antibody (1:200 dilution, 2.5 µg/mL), a rabbit anti-OCT4 antibody (1:500 dilution, 400 ng/mL; C-10; sc-5279, Santa Cruz Biotechnology), or a mouse anti-CDX2 antibody (1:500 dilution, 100 µg/mL; CDX-88; BioGenex, San Ramon, CA). Second antibody was used; an HRP-conjugated anti-mouse secondary antibody (1:2000 or 1:10000 dilution; GE Healthcare UK Ltd, Little Chalfont, UK) or an HRP-conjugated anti-rabbit secondary antibody (1:1000 or 1:2000 dilution; GE Healthcare UK Ltd) in TBS-T for 1 h at RT. The membrane was extensively washed three times with TBS-T, and then bound antibodies were detected using the Enhanced Chemiluminescence (ECL) system (GE Healthcare UK Ltd). α-TUBULIN was used as an internal control.

TUNEL assay

Apoptotic cells in embryos at E3.5, E4.5 and E5.5, and outgrowth embryos at E6.5 and E7.5 were identified with the *In Situ* Cell Death Detection Kit (Roche Diagnostics Corp., Indianapolis, IN) using the protocol recommended by the manufacturer.

Statistical Analysis

All data were expressed as the mean ± SEM. Statistical analysis of the date was performed by analysis of variance (ANOVA) with Student's t-test for comparing two groups.

Ethical Approval for the Use of Animals

All animal experiments were approved by the Animal Research Committee of Kyoto University (Permit Number: 24-17) and were performed in accordance with the committee's guidelines.

Results

Expression of the *Smyd3* mRNA and protein in mouse preimplantation embryos

First, we revealed the expression pattern of *Smyd3* mRNA and the localization of SMYD3 protein in mouse preimplantation embryos. qRT-PCR analysis of *Smyd3* mRNA in preimplantation embryos indicated that the expression levels increased after the 1-cell stage, peaked at the 4-cell stage, and then slightly decreased until the blastocyst stage (Fig. 1A). Furthermore, immunostaining showed that SMYD3 dominantly localized to the nuclei from the 2-cell to the 8-cell stage (Fig. 1B).

Effects of *Smyd3* knockdown on the development of mouse embryos

In order to investigate the role of *Smyd3* in early embryonic development, we knocked down the expression of *Smyd3* in mouse preimplantation embryos. Embryos injected with siRNA targeting *Smyd3* (si*Smyd3*-1 and si*Smyd3*-2) were cultured until E5.5. qRT-PCR and immunoblotting showed that the reduction in the expression of *Smyd3* mRNA and protein was observed at the blastocyst stage (Fig. 2A and B; Supplementary Fig. 1A). Additionally, Immunostaining showed that SMYD3 protein levels were also reduced from the 1-cell stage and continued through the blastocyst stage (Fig. 2C). Nevertheless, no differences between *Smyd3*-knockdown and control embryos were noted with respect to morphology or percentage of embryonic development up to E5.5 (Fig. 3A and B; Supplementary Fig. 1B and C). Cell numbers in

E4.5 blastocysts were counted after OCT4 (ICM) and CDX2 (TE) staining. The data demonstrated that *Smyd3*-knockdown blastocysts had normal numbers of cells in both populations (Fig. 3C). To examine the pluripotency of *Smyd3*-knockdown embryos, outgrowth experiments were performed on E3.5 embryos. In control embryos, the percentages of successful attachment and ICM-derived colony formation were 88.9% and 81.1%, respectively, while in *Smyd3*-knockdown embryos, the percentages were 71.4% and 45.8 %, respectively (Fig. 3D and E). In addition, to test the viability of *Smyd3*-knockdown embryos in vivo, si*Smyd3*-1-injected embryos were transferred into the oviducts of pseudopregnant mice. The percentage of offspring derived from *Smyd3*-knockdown embryos was significantly reduced as compared to controls (Table 1).

Knockdown of *Smyd3* leads to the down-regulation of linage-specific genes

In order to analyze the reasons of the defect of peri-implantation embryonic development, we performed qRT-PCR on 4- and 8-cell stage embryos for *Smyd3* and early differentiation markers such as *Oct4* and *Cdx2*. In addition, to confirm the levels of H3K4me3 in *Smyd3*-knockdown embryos at the 4- and 8-cell stages immunostaining was performed. The results demonstrated that the expression of *Smyd3* mRNA was significantly decreased in *Smyd3*-knockdown embryos (Fig. 4A); however, H3K4me3 levels remained unchanged (Fig. 4B).

Interestingly, the transcription of *Oct4* at the 4-cell and 8-cell stages and of *Cdx2* at the 8-cell stage was significantly decreased in *Smyd3*-knockdown embryos (Fig. 4C and D). To further investigate the influence of the reduced expression of *Smyd3*, *Oct4*, and *Cdx2* at the 4- and 8-cell stages, we performed qRT-PCR on E4.5 blastocyst stage embryos for *H2afz*—an internal control—and lineage-specific genes, e.g., the ICM/EPI markers, *Oct4*, *Nanog*, and *Sox2*; the PE marker, *Gata6*; and the TE markers, *Cdx2* and *Eomes*. The results demonstrated that the transcription levels of *Oct4*, *Nanog*, *Sox2*, *Gata6*, *Cdx2*, and *Eomes* were all significantly decreased in *Smyd3*-knockdown embryos at the blastocyst stage, while *H2afz* transcript levels remained unchanged (Fig. 5A; Supplementary Fig. 2A). Furthermore, the protein levels of OCT4, CDX2, NANOG, SOX2, GATA6, and EOMES were confirmed on E4.5 blastocysts. The results demonstrated that the levels of OCT4, CDX2, NANOG, SOX2, GATA6, and EOMES were significantly reduced in *Smyd3*-knockdown embryos at the blastocyst stage (Fig. 5B; Supplementary Fig. 2B and 3A). Additionally, global H3K4me3 levels also remained unchanged in the treated embryos at the blastocyst stage (Supplementary Fig. 3B).

Knockdown of *Smyd3* does not induce apoptosis during peri-implantation development

Since it has been reported that *Smyd3*-knockdown induces apoptosis in human cancer cells (Chen, et al. 2007), we investigated the effects of *Smyd3*-knockdown on apoptosis in mouse peri-implantation embryos. However, there was no differences in the number of TUNEL-positive cells between *Smyd3*-knockdown and control embryos (Fig. 6).

Discussion

SMYD3 methylates both H3K4 and H4K5 (Hamamoto, et al. 2004, Van Aller, et al. 2012), recruits RNA polymerase II through an RNA helicase to form a transcription complex, and elicits its oncogenic effects by activating the transcription of downstream target genes (Hamamoto, et al. 2004, Hamamoto, et al. 2006, Liu, et al. 2007, Liu, et al. 2013). SMYD3 is also involved in apoptosis and the inhibition of cell growth, migration, and invasion (Xu, et al. 2006, Zou, et al. 2009). Here, we observed *Smyd3* mRNA expression patterns and protein localization during mouse preimplantation development, and showed that *Smyd3* knockdown led to a defect in their ability to attach to a matrix and outgrowth *in vitro*, and to a reduction in the numbers of viable offspring, suggesting that SMYD3 has important roles during peri-implantation development.

Previous reports have shown that *Oct4* and *Sox2* form an *Oct4*/*Sox2* complex and bind

238 directly to their own promoter regions in embryonic stem (ES) cells (Chew, et al. 2005,
239 Okumura-Nakanishi, et al. 2005). *Oct4*, *Nanog*, and *Sox2* are reported to be regulated via the
240 *Oct4*/*Sox2* complex, and to form a self-reinforcing regulatory loop in ES cells (Chew, et al. 2005,
241 Mitsui, et al. 2003, Okumura-Nakanishi, et al. 2005, Rodda, et al. 2005). In mouse preimplantation
242 embryos, *Oct4*, *Nanog*, and *Sox2* are required for the maintenance of ICM pluripotency (Avilion, et
243 al. 2003, Mitsui, et al. 2003, Nichols, et al. 1998). Additionally, *Nanog* is known to negatively
244 interact with *Gata6* and both genes are known as key regulators in the establishment of EPI and PE
245 fates, respectively (Frankenberg, et al. 2011, Kang, et al. 2013, Morris, et al. 2010, Schrode, et al.
246 2014). *Nanog* deficient mouse embryos are arrested during post-implantation development due to
247 widespread expression of *Gata6* in the EPI (Frankenberg, et al. 2011, Mitsui, et al. 2003). By
248 contrast, *Gata6* deficient mouse embryos are arrested during post-implantation development due to
249 widespread expression of *Nanog* in the PE (Morris, et al. 2010, Schrode, et al. 2014). In the present
250 study, we showed that *Smyd3* knockdown led to the suppression of the embryonic transcription of
251 *Oct4* from the 4-cell stages. In addition, we also showed that, in blastocysts, *Smyd3* knockdown
252 abrogates the transcription of other ICM and EPI markers, e.g., *Nanog* and *Sox2*; and of PE
253 markers, such as *Gata6*. Therefore, it is possible that the lack of pluripotency genes, such as *Oct4*,
254 *Nanog*, and *Sox2*; and of PE-specific genes, such as *Gata6*, could account for the defects observed

in *Smyd3*-knockdown embryos, including poor outgrowth *in vitro* and a reduction in the numbers of viable offspring. Furthermore, *Cdx2* and *Eomes* are reported to be essential for the specification and differentiation of TE (Russ, et al. 2000, Strumpf, et al. 2005). *Cdx2*-knockout embryos fail to hatch from the zona pellucida or to implant *in vivo*, and also fail to attach to matrix substrates *in vitro*, even when the zona pellucida has been removed (Strumpf, et al. 2005). In the present study, we showed that *Smyd3* knockdown led to the suppression of the embryonic transcription of *Cdx2* from the 8-cell stages. Additionally, we also showed that, in blastocysts, *Smyd3* knockdown abrogates the transcription of TE markers, such as *Eomes*. Therefore, it is possible that the suppression of TE-specific genes, such as *Cdx2* and *Eomes*, could account for a defect in their ability to attach to a matrix substrate *in vitro* observed in *Smyd3*-knockdown embryos. Furthermore, it has been reported that *Smyd3*-knockdown induces apoptosis in cancer (Chen, et al. 2007). In the present study, however, we showed that the number of apoptotic cells was not increased in *Smyd3*-knockdown embryos. Together, these observations also suggested that SMYD3 plays an important role in peri-implantation embryonic development via the activation of lineage-specific genes expression. However, we demonstrated that in *Smyd3*-knockdown embryos global H3K4me3 levels appeared unchanged and that developmental arrest did not occur up to the blastocyst stage, even though *Smyd3* knockdown suppressed the nuclear localization of SMYD3 protein at ZGA and MGA.

Smyd3 tri-methylates H3K4 and some chromatin remodeling complexes are recruited to H3K4me3 (He, et al. 2005, Sims, et al. 2005). These chromatin remodeling factors are involved in an open chromatin state, which correlates with a globally permissive transcriptional state. Within this 'loose' chromatin state, transcription factors can bind to the promoter regions of genes and activate transcription. The decreased expression of lineage-specific genes, such as *Oct4*, *Nanog*, and *Cdx2*, that we observed in this study echos results obtained in *Klf5*-null embryos (Lin, et al. 2010) and in *Hmgpi*-knockdown embryos (Yamada, et al. 2010), suggesting that SMYD3 functions via these transcription factors to determine lineage specificity. Previous studies showed that MLL2, one of the H3K4 methyltransferases, affects the global H3K4me3 levels at ZGA (Andreu-Vieyra, et al. 2010), and the SETD1A/SETD1B methyltransferase complex that modifies H3K4 affects the global H3K4me3 levels at MGA (Bi, et al. 2011). Additionally, previous studies demonstrated that a knockdown of SMYD3 expression leads to the selective decrease in H3K4 methylation levels on oncogene promoter regions in cancer cells (Cock-Rada, et al. 2012, Liu, et al. 2013, Medjkane, et al.). From these observations it is possible that H3K4me3 level depends on the type of methyltransferase and that SMYD3 modified H3K4 within the promoter regions of the lineage-specific genes. In addition, a recent report demonstrated that while global levels of H3K4me3 do not change upon the loss of *Smyd3* in the human breast carcinoma cell line, MCF7,

289 the global levels of H4K5me, a novel chromatin target of *Smyd3*, do change (Van Aller, et al. 2012).
290 This result indicates that SMYD3 is required for H4K5 methylation in cancer cells. It is also
291 possible that SMYD3 is involved in the regulation of genes in mouse preimplantation embryos
292 through the methylation of H4K5.

293 In summary, our results demonstrated that *Smyd3*-knockdown does not have a critical
294 effect on early embryonic development or on global H3K4me3 levels, but that it does play an
295 important role in early embryonic lineage commitment and peri-implantation development by
296 regulating the expression of *Oct4* and *Cdx2* at MGA. Accordingly, we demonstrated the importance
297 of *Smyd3* as a key regulator of lineage-specific genes in mouse preimplantation embryos.

298

299 **Declaration of interest**

300 The authors declare that there is no conflict of interest that could be perceived as
301 prejudicing the impartiality of the research reported.

302

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Figure Legends

Figure 1. *Smyd3* expression and the localization of SMYD3 in preimplantation embryos.

(A) qRT-PCR analysis of *Smyd3* expression in preimplantation embryos. The expression levels at each developmental stage were normalized by using *H2afz* as an internal control. Data are expressed as mean ± SEM (n=3). (B) Localization of *Smyd3* in mouse preimplantation embryos. SMYD3 (red) is detected by immunofluorescence and nuclei (blue) are stained with Hoechst dye.

Figure 2. Reduction in the expression of *Smyd3* mRNA and protein at the blastocyst stage.

(A) qRT-PCR analysis of *Smyd3* mRNA at the blastocyst stage in *Smyd3*-knockdown and control embryos (*$p < 0.05$). The expression levels were normalized by using *Gapdh* as an internal control. Data are expressed as mean ± SEM (n=3). (B) Immunoblot analysis of SMYD3 and α-TUBULIN in *Smyd3*-knockdown and control blastocyst embryos. (C) Immunostaining of SMYD3 protein in *Smyd3*-knockdown and control blastocyst embryos (red, SMYD3; blue, chromatin).

Figure 3. The effects of *Smyd3* knockdown on pre- and post-implantation development.

(A) Pairs of representative photos showing the development of preimplantation embryos injected with either si*Smyd3*-1 or siControl. Embryos were photographed at 36 h after in vitro fertilization and 24 h intervals thereafter. (B) The percentage of development at E1.5 (≥ 2-cell), E2.5 (≥ 4-cell), E3.5 (≥ morula), E4.5 (≥ blastocyst), and E5.5 (≥ hatching) in *Smyd3*-knockdown and control embryos. Data are expressed as mean ± SEM (n=3). Twenty to 25 embryos were used in each experiment (63 and 69 embryos in total in siControl and si*Smyd3*-1, respectively). (C) The numbers of ICM and TE cells were assessed by counting OCT4-positive cells and CDX2-positive cells, respectively. Total embryonic cell numbers were obtained by combining the numbers of ICM and TE cells. Data are expressed as mean ± SEM (n=16). (D) Photographs depict representative results of outgrowth experiments for control and *Smyd3*-knockdown embryos (ICM-derived colony, arrowhead; trophectoderm cells, arrow). (E) The successful percentages of attachment and ICM-derived colony formation in *Smyd3*-knockdown and control embryos after 4 days in culture (*$p < 0.05$). Data are expressed as mean ± SEM (n=6). Thirteen to 83 embryos were used in each experiment (238 and 243 embryos in total in siControl and si*Smyd3*-1, respectively).

Figure 4. SMYD3 regulates the expression of *Oct4* and *Cdx2* at MGA.

(A) qRT-PCR analysis of *Smyd3* mRNA in *Smyd3*-knockdown and control embryos at the 4- and

8-cell stages (*$p < 0.05$). Expression levels were normalized to *Gapdh* as an internal control. Data are expressed as mean ± SEM (n=3). (B) Immunostaining of H3K4me3 in *Smyd3*-knockdown and control embryos at the 4- and 8-cell stages (red, SMYD3; green, H3K4me3; blue, chromatin). (C and D) qRT-PCR analysis of *Oct4* mRNA and *Cdx2* mRNA in *Smyd3*-knockdown and control embryos at the 4- and 8-cell stages (*$p < 0.05$). Data are expressed as mean ± SEM (n=3).

Figure 5. The effects of *Smyd3* knockdown on the expression of lineage-specific genes in blastocysts.

(A) qRT-PCR analysis of the early-lineage markers *Oct4*, *Nanog*, *Sox2*, *Cdx2*, *Eomes*, *Gata6*, and *H2afz* in *Smyd3*-knockdown and control blastocyst embryos (*$p < 0.05$). The expression levels were normalized by using *Gapdh* as an internal control. Data are expressed as mean ± SEM (n=3). (B) Immunostaining of OCT4, CDX2, NANOG, SOX2, GATA6, and EOMES in *Smyd3*-knockdown and control blastocyst embryos (OCT4, red; CDX2, green; NANOG, red; SOX2, red; GATA6, red; EOMES, green; chromatin, blue).

Figure 6. The effects of *Smyd3*-knockdown on the induction of apoptosis.

TUNEL-positive cells (green nuclei) were detected. Photographs depict representative results of

TUNEL assay for control and *Smyd3*-knockdown embryos before (E3.5-E5.5) and after (E6.5-E7.5) outgrowth. Nuclei are stained with Hoechst dye (blue).

Supplementary Figure 1. The effects of si*Smyd3*-2 injection on preimplantation development. (A) qRT-PCR analysis of *Smyd3* mRNA at the blastocyst stage in embryos injected with either si*Smyd3*-2 or siControl (*$p < 0.05$). The expression levels were normalized by using *Gapdh* as an internal control. Data are expressed as mean ± SEM (n=3). (B) Pairs of representative photos showing the development of preimplantation embryos injected with either si*Smyd3*-2 or siControl. Embryos were photographed at 36 h after in vitro fertilization and 24 h intervals thereafter. (C) The percentage of development at E1.5 (≥ 2-cell), E2.5 (≥ 4-cell), E3.5 (≥ morula), E4.5 (≥ blastocyst), and E5.5 (≥ hatching) in embryos injected with either si*Smyd3*-2 or siControl. Data are expressed as mean ± SEM (n=3). Twenty to 30 embryos were used in each experiment (75 and 88 embryos in total in siControl and si*Smyd3*-2, respectively).

Supplementary Figure 2. The effects of si*Smyd3*-2 injection on the expression of *Oct4* and *Cdx2*. (A) qRT-PCR analysis of the early-lineage markers *Oct4* and *Cdx2* in blastocyst embryos injected with either si*Smyd3*-2 or siControl (*$p < 0.05$). The expression levels were normalized by using

Gapdh as an internal control. Data are expressed as mean ± SEM (n=3). (B) Immunostaining of OCT4 and CDX2 in blastocyst embryos injected with either si*Smyd3*-2 or siControl (OCT4, red; CDX2, green; chromatin, blue).

Supplementary Figure 3. Knockdown of *Smyd3* influence the levels of OCT4 and CDX2 proteins not the levels of H3K4me3.

(A) Immunoblot analysis of OCT4, CDX2, H3K4me3, and α-TUBULIN in *Smyd3*-knockdown and control blastocyst embryos. (B) Immunostaining of H3K4me3 in *Smyd3*-knockdown and control blastocyst embryos (H3K4me3, green; chromatin, blue).

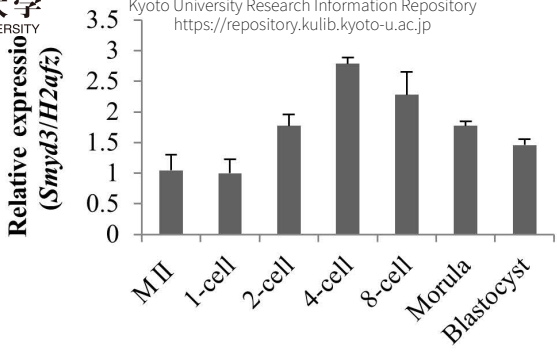
Table 1. Effect of *Smyd3*-knockdown on mouse embryo development.

	No. trials (No. recipients)	No. embryos transferred	No. pregnant mice	No. live offspring (mean ± SEM)	% live offspring
siControl	4	60[a]	4	31 (7.8±0.25)	51.7[b]
si*Smyd3*-1	4	60[a]	4	12 (3.0±0.41)	20.0[c]

[a] Fifteen embryos were transferred to each recipient at the trial.

[b, c] $p < 0.05$

F



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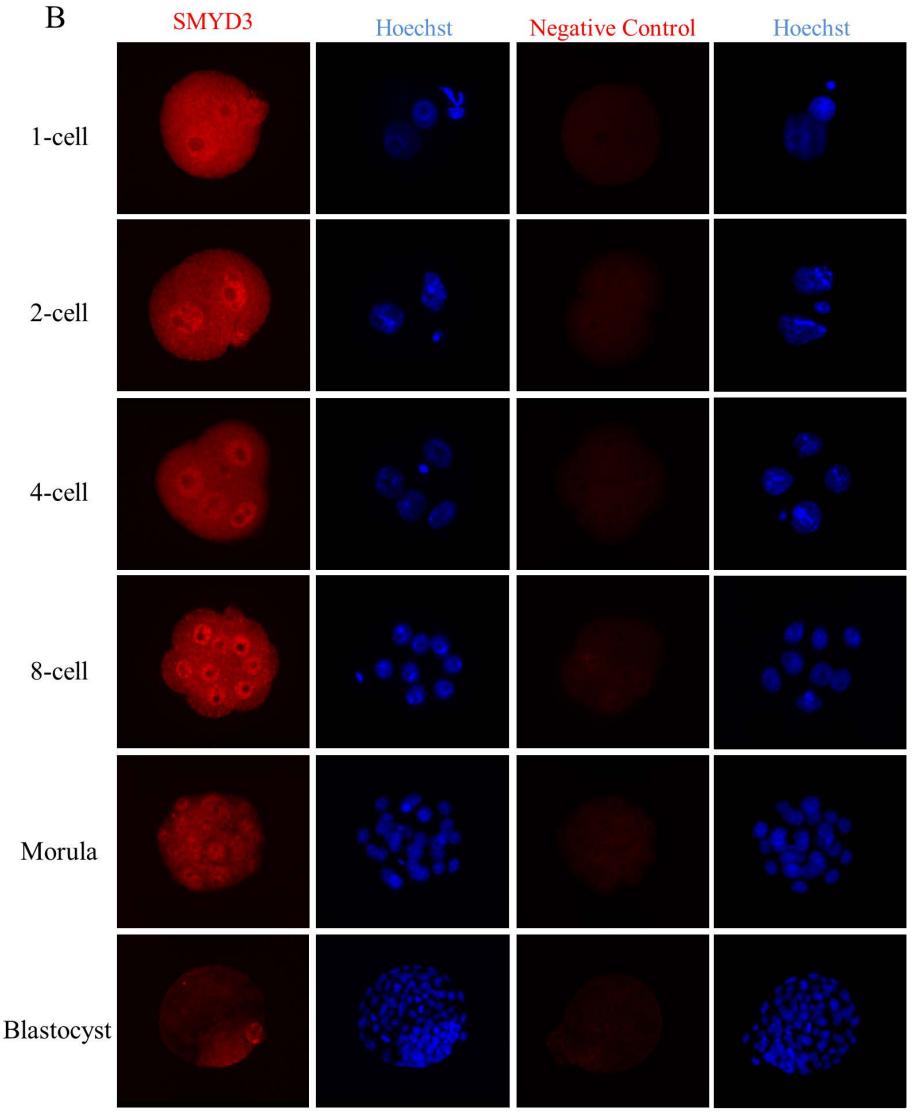

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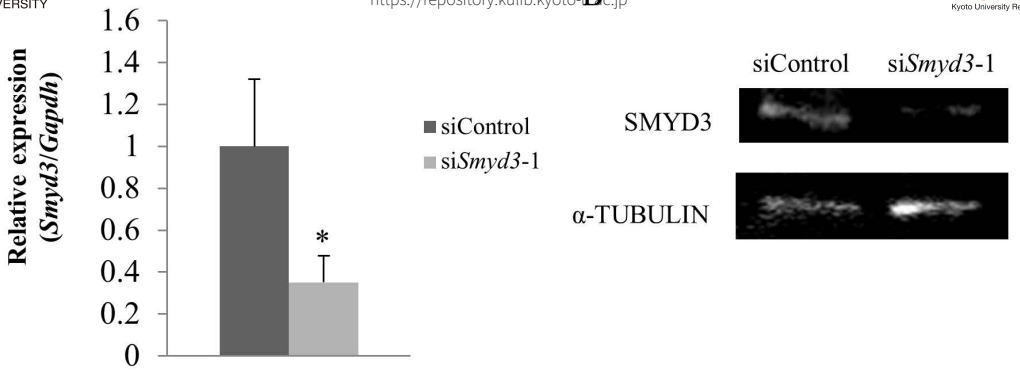

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